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BIOLOGY CONTRIBUTION

FIVE-CHLORODEOXYCYTIDINE, A TUMOR-SELECTIVE ENZYME-DRIVEN RADIOSENSITIZER, EFFECTIVELY CONTROLS FIVE ADVANCED HUMAN TUMORS IN NUDE MICE

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Purpose: The study's goals were as follows: (*1*) to extend our past findings with rodent tumors to human tumors in nude mice, (*2*) to determine if the drug protocol could be simplified so that only CldC and one modulator, tetrahydrouridine (H_4U), would be sufficient to obtain efficacy, (*3*) to determine the levels of deoxycytidine kinase and dCMP deaminase in human tumors, compared to adjacent normal tissue, and (*4*) to determine the effect of CldC on normal tissue radiation damage to the cervical spinal cord of nude mice.

Methods and Materials: The five human tumors used were as follows: prostate tumors, PC-3 and H-1579; glioblastoma, SF-295; breast tumor, GI-101; and lung tumor, H-165. The duration of treatment was 3–5 weeks, with drugs administered on Days 1–4 and radiation on Days 3–5 of each week. The biomodulators of CldC were N-(Phosphonacetyl)-L-aspartate (PALA), an inhibitor of aspartyl transcarbamoylase, 5-fluorodeoxycytidine (FdC), resulting in tumor-directed inhibition of thymidylate synthetase, and H_4U, an inhibitor of cytidine deaminase. The total dose of focused irradiation of the tumors was usually 45 Gy in 12 fractions.

Results: Marked radiosensitization was obtained with CldC and the three modulators. The average days in tumor regrowth delay for X-ray compared to drugs plus X-ray, respectively, were: PC-3 prostate, 42–97; H-1579 prostate, 29–115; glioblastoma, 5–51; breast, 50–80; lung, 32–123. Comparative studies with PC-3 and H-1579 using CldC coadministered with H_4U, showed that both PALA and FdC are dispensable, and the protocol can be simplified with equal and possibly heightened efficacy. For example, PC-3 with X-ray and (*1*) no drugs, (*2*) CldC plus the three modulators, (*3*) a high dose of CldC, and (*4*) escalating doses of CldC resulted in 0/10, 3/9, 5/10, and 6/9 cures, respectively. The tumor regrowth delay data followed a similar pattern. After treating mice only 1½ weeks with CldC + H_4U, 92% of the PC-3 tumor cells were found to possess CldU in their DNA. The great majority of head-and-neck tumors from patient material had markedly higher levels of dC kinase and dCMP deaminase than found in adjacent normal tissue. Physiologic and histologic studies showed that CldC + H_4U combined with X-ray, focused on the cervical spinal cord, did not result in damage to that tissue.

Conclusions: 5-CldC coadministered with only H_4U is an effective radiosensitizer of human tumors. Ninety-two percent of PC-3 tumor cells have been shown to take up ClUra derived from CldC in their DNA after only 1½ weeks and 2 weeks of bolus i.p. injections. Enzymatic alterations that make tumors successful have been exploited for a therapeutic advantage. The great electronegativity, coupled with the relatively small Van der Waal radius of the Cl atom, may result in CldC's possessing the dual advantageous properties of FdC on one hand and BrdU and IdU on the other hand. These advantages include autoenhancing the incorporation of CldUTP into DNA by not only overrunning but also inhibiting the formation of competing TTP pools in tumors. A clinical trial is about to begin, with head-and-neck tumors as a first target of CldC radiosensitization. © 2001 Elsevier Science Inc.

5-chloro-2′-deoxycytidine, Human tumors of the prostate, Brain, Lung and breast, Radiation therapy, Radiosensitization.

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This paper is dedicated to the memory of Dr. Stephen Zamenhof, the postdoctoral mentor and source of inspiration to S. Greer, and to the memory of Stanley Marion, a devoted and skilled veterinary technician.

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INTRODUCTION

The discovery of 5-bromouracil radiosensitization by Greer and Zamenhof (1) and Greer (2) ignited the hope that the control of solid human tumors was close at hand. Confirming and extending studies from the laboratories of Djordjevic and Szybalski (3), Kaplan and Tomplin (4), and Bagshaw *et al.* (5) was followed by intensive research activity. The halogenated analogs of deoxyuridine did provide clinically relevant, though modest, radiosensitization of human tumors that were poorly radiation responsive, including unresectable sarcomas, high-grade gliomas, astrocytomas, and liver metastases from colorectal carcinomas in Phase I/II studies (6–17). However, after many years of clinical studies, the reality is that 5-bromodeoxyuridine and 5-iododeoxyuridine combined with radiotherapy are no more effective than other modalities of therapy against glioblastomas (11). The foregoing is the reason why it is compelling to emphasize the critical differences between 5-chlorodeoxycytidine and the 5-halogenated analogs of deoxyuridine as the quest continues for an effective radiosensitizer of human tumors. The substantial superiority of 5-chlorodeoxycytidine over 5-iododeoxyuridine as a radiosensitizer was demonstrated in our past study with the rodent mammary adenocarcinoma EMT-6 (18). Lawrence *et al.* showed a greater or equal incorporation of IdU into DNA of bone marrow and intestine of nude mice compared to a human colon tumor (19). These results on the tissue distribution of IdU were confirmed and extended by Kinsella *et al.* (20). Instead of relying on rapid cell kinetics, which is the mode of radiosensitization by BrdU and IdU (a property also displayed by bone marrow and intestine), the technology using CldC depends on the elevation of deoxycytidine kinase (21–27) and/or dCMP deaminase (28–31) in human tumors for a therapeutic advantage (see Fig. 1).

In the present study, the major aim was to extend our past findings with rodent tumors (18) to tumor inhibition experiments with five human tumors in nude mice. These human tumors were two prostates, one breast, one lung, and one glioblastoma. Based on results from this enzyme-driven, tumor-selective radiosensitization, this technology is moving toward a Phase I/II clinical trial with the participation of the National Cancer Institute of the National Institutes of Health.

METHODS AND MATERIALS

Drugs

N-(Phosphonacetyl)-L-aspartic acid and tetrahydrouridine (H_4U) were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (National Institutes of Health, Bethesda, MD). FdC and CldC were obtained by custom synthesis (Ash-Stevens, Detroit, MI); 5-iododeoxyuridine was obtained from Sigma (St. Louis, MO).

Human tumor lines, animals

PC-3, a human prostate adenocarcinoma, was originally obtained from the American Type Culture Collection (Rockville, MD) and has been maintained by the Rumbaugh-Goodwin Institute for Cancer Research (RGI) since 1994. GI-101, an estrogen-independent metastatic human breast carcinoma derived from an infiltrating ductal breast carcinoma, has been maintained at the RGI since 1985 and has been described in detail (32). The three human tumors obtained from the Division of Cancer Treatment and Diagnosis, National Cancer Institute (National Institutes of Health, Frederick, MD) are as follows: MRI-H-1579, an adenocarcinoma of the prostate; SF-295, a glioblastoma; and MRI-H-165, a squamous cell carcinoma of the lung. In all studies, two 2-mm fragments were s.c. implanted with trochars unilaterally into the right flank region above the hip. In every experiment, animals were implanted with tumors only two passages away from the initial source. The nude mice (BALB/c, nu/nu) were obtained from Harlan Laboratories (Indianapolis, IN) and weighed 22–26 grams at the start of each experiment. Males were used for the experiments with prostate tumors, and females for the breast, glioblastoma, and lung. For the experiments with PC-3 and H-165 that demonstrated the efficacy of the simplified protocol, and for studies examining normal tissue damage, the nude mice were obtained from the RGI.

Efforts were made to use mice with tumors with an initial volume no greater than 300 mm^3 and no less than 100 mm^3. The initial day of treatment varied with each tumor, depending on its rate of growth. The experiment with the rapidly growing glioblastoma began on Day 13 postimplantation (PI), whereas the experiment with the slow-growing breast tumor began on Day 45 PI.

Human cell cultures SCC-1 and SCC-6, with which the levels of dC kinase and dCMP deaminase were determined, were obtained from Dr. Thomas E. Carey (University of Michigan).

Drug treatment and radiation

The treatment schedule using four drugs is shown in Table 1. All drugs were sterile filtered and administered i.p. H_4U was always coadministered in the same syringe as the appropriate nucleoside (FdC or CldC).

The radiation source for all the studies, except for the experiments with glioblastoma, was a Siemens (Islen, NJ) Stabilipan 250 kVp orthovoltage therapy unit (15 mA, 4-mm Al filter producing 144.24 cGy/min at 70 cm). Six animals were irradiated at the same time with the collimator fully open. Animals were anesthetized by i.p. injection with ketamine/xylazine. Mice were shielded with 3-mm-thick lead shields with the tumor protruding. Tumor growth was measured in three dimensions using calipers. The product of three dimensions × 0.5 was plotted vs. days postimplantation. Tumor measurements and weights of the mice were determined no less than twice per week. The standard error of the mean was plotted for the prostate tumors and for weight loss data for the experiments involving lung and

Table 1. The standard protocol (StP), indicating the bolus doses of i.p.-administered drugs and the schedule of the administration of the drugs and radiation. This protocol was followed for 3 to 5 weeks as indicated in Table 2.

Time	Mon.	Tues.	Wed.	Thurs.	Fri.
9 am	PALA (70)*	FdC + H_4U (7) (25)	CldC + H_4U (150) (25)	CldC + H_4U (125) (25)	
1 pm	FdC + H_4U (7) (25)				Radiation
3 pm			Radiation	Radiation	
4 pm		CldC + H_4U (180) (25)			
5 pm	CldC + H_4U (180) (25)				

* (mg/kg)

Abbreviations: PALA, N-(Phosphonacetyl)-L-aspartate; FdC, 5-fluoro-2′-deoxycytidine; CldC, 5-chloro-2′-deoxycytidine; H_4U: tetrahydrouridine.

breast tumors. For the experiments with breast, as well as comparative experiments with PC-3, the data were found to be best represented by plotting the volumes of individual tumors.

Treatment of the glioblastoma was undertaken with the linear accelerator, because the 250-kV source was not available. Tumors were irradiated with 4-MV X-rays from a Varian Clinac 4 linear accelerator using a 30 × 30 cm^3 field at a distance of 80 cm (dose rate 3–3.5 Gy/min) with tumors positioned around the periphery of the radiation field such that each tumor was in the field while the remainder of the animal was shielded (outside the field). The sharp penumbra at the edge of the field allows a full dose to the tumor and overlying skin, with little dose to the rest of the animal. A 1-cm-thick layer of bolus material (water and gelling agent, wrapped in plastic) was placed over each tumor; consequently, the tumor was located between 1.0 (Dmax for 4-MV X-ray) and 1.5 cm depth; resultant dose uniformity over the tumor was ±1%. With this system, we irradiated 12–14 mice at one time.

Enzymatic studies

The methods described by Dobersen and Greer (33) were used for the assay of deoxycytidine kinase and dCMP deaminase. H_4U was added to the reaction mixture for the dC kinase assay. Two different solvent systems were used for the dCMP deaminase assay. DEAE cellulose thin layer chromatography sheets (TLC) (Baker, Phillipsburg, NJ), with 0.01 N HCl as the solvent, separated ^{3}H-dCMP from ^{3}H-dUMP after 30 min with dCMP closer to the solvent front (Rf dCMP: 0.47; Rf dUMP: 0.21). The more desirable separation occurred with isopropanol:H_2O:HCl at a ratio of 4:2:1 after 7 h using Analtech (Newark, DE) Cellulose TLC. The product, ^{3}HdUMP, was close to the solvent front with this chromatographic system (Rf dCMP: 0.85; Rf dUMP: 0.96).

Tissue was obtained in accordance with the ethical standards of the responsible institutional committee on human experimentation.

Normal tissue damage

In view of our interest in treating head-and-neck cancer patients, we undertook a study to determine whether CldC + H_4U would affect radiation damage to the spinal cord, which is often at risk in the treatment of head-and-neck cancer patients with radiotherapy. Nude mice, untreated or treated with escalating doses of CldC and H_4U (Schedule D in "Results"), were irradiated with 2.5 Gy or 4 Gy per radiation dose in the cervical spinal cord region for a total dose of 30 or 48 Gy, respectively. The mice were anesthetized and restrained in plastic jigs. The mice were protected by a 3-mm-thick lead shield with a 14-mm × 4-mm slit placed over the cervical region of the spine.

Mice were killed by deep inhalation of anesthesia with methoxyflurane (metofane) and decapitated. The calvarium was removed, the brain was separated from the cervical-medullary junction, and the brain was removed. The spinal column with proximal ribs was removed by the posterior approach. A thoraco-abdominal incision was made, and the systemic organs, including heart, lung, liver, spleen, kidneys, and intestine, were removed. These tissues were immersed in 500 mL of 10% buffered formalin.

After a 3-day fixation period, the cervical and upper thoracic spinal cord was removed after careful chipping-removal of the vertebral arches. Between 4 and 7 pieces of cervical and upper thoracic cord, measuring approximately 0.3 cm in length, were dehydrated, embedded in paraffin for cross-sectional analysis, cut at 6 μ, and stained with hematoxylin-eosin.

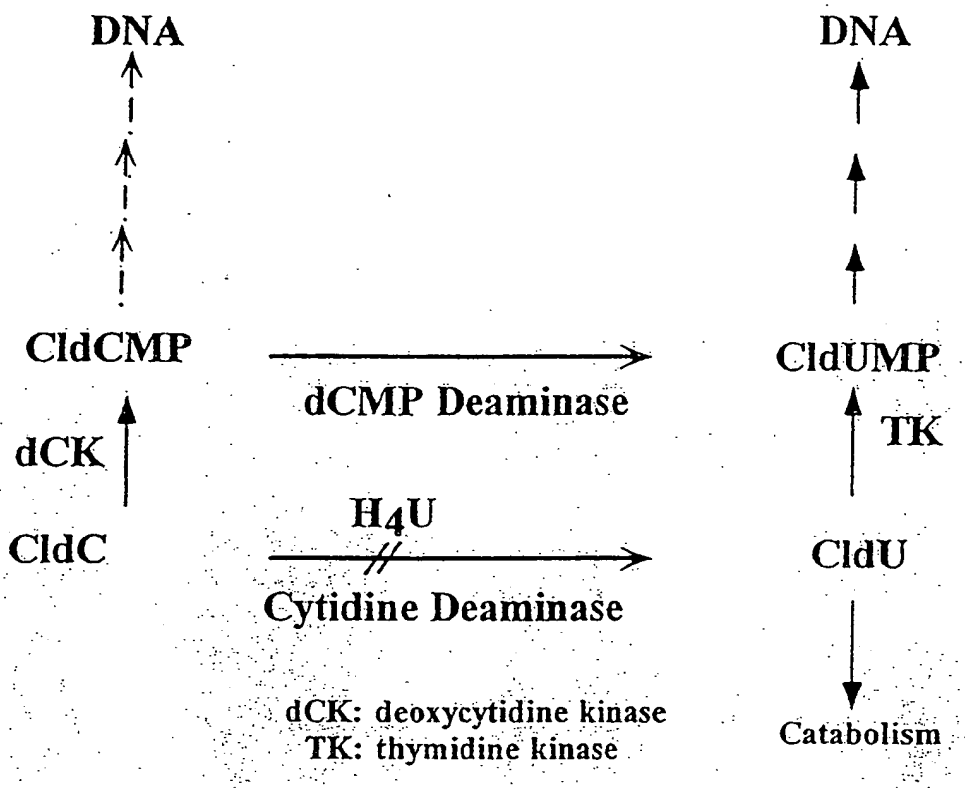
Microscopic examination: All sections were examined without knowledge of the experimental condition.

All animal studies were conducted in accordance with the National Research Council's guide for the care, treatment, and use of laboratory animals.

RESULTS

Human prostate tumor PC-3

Figure 2 summarizes an experiment in which the mice were treated with drugs for 4 weeks using the standard



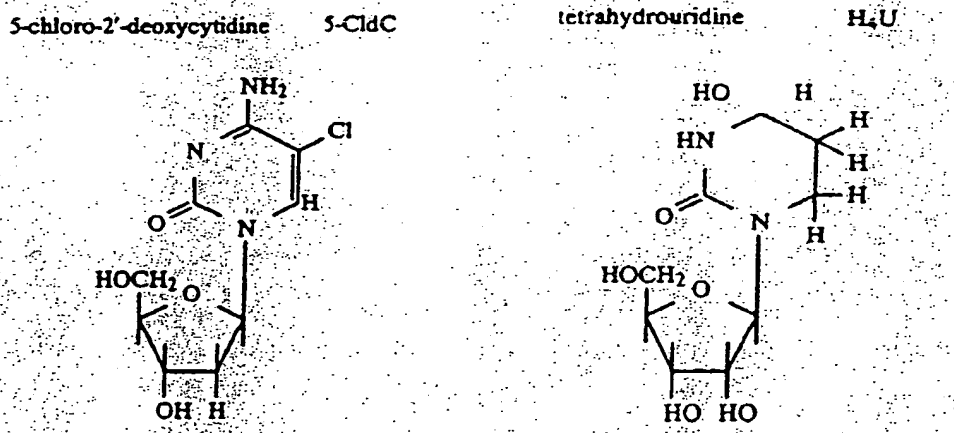


Fig. 1. The pathway of metabolism of 5-chloro-2'-deoxycytidine. The broken arrows reflect the poor affinity of dCMP kinase and the higher kinases for their substrates. Also included are the chemical structures of CldC and H_4U.

protocol described in "Methods and Materials" (Table 1). Treatment began 35 days PI and ended 61 days PI. The total dose was 47 Gy in 11 fractions. Administration of the full treatment (i.e., drugs [CldC and modulators] and radiation) resulted in 9/10 (90%) fully controlled tumors (<25 mm^3) for 45 days after cessation of treatment. In contrast, treatment with radiation alone resulted in 9/9 (100%) recurrences in the same period of time (45 days after treatment ended) with 0/9 tumors less than 25 mm^3 at any point in the study. As shown in Table 2, the days in tumor regrowth delay for drugs, X-ray, drugs plus X-ray, and drugs plus X-ray excluding N-(Phosphonacetyl)-L-aspartate (PALA) were 0, 52, >97, and >113, respectively, with a cure assigned a value of >200 days.

Human prostate tumor H-1597

An experiment in which mice bearing another prostate tumor were treated for 4 weeks, as described for PC-3, is shown in Fig. 3. The total dose was 43 Gy in 11 fractions. Treatment began on Day 18 PI and ceased on Day 41 PI. This tumor was the most sensitive to drug treatment among the five human tumors in this study. On Day 80 PI, 25 days after the cessation of treatment, 1/8 (13%) and 6/7 (86%) tumors were less than 75 mm^3 in the

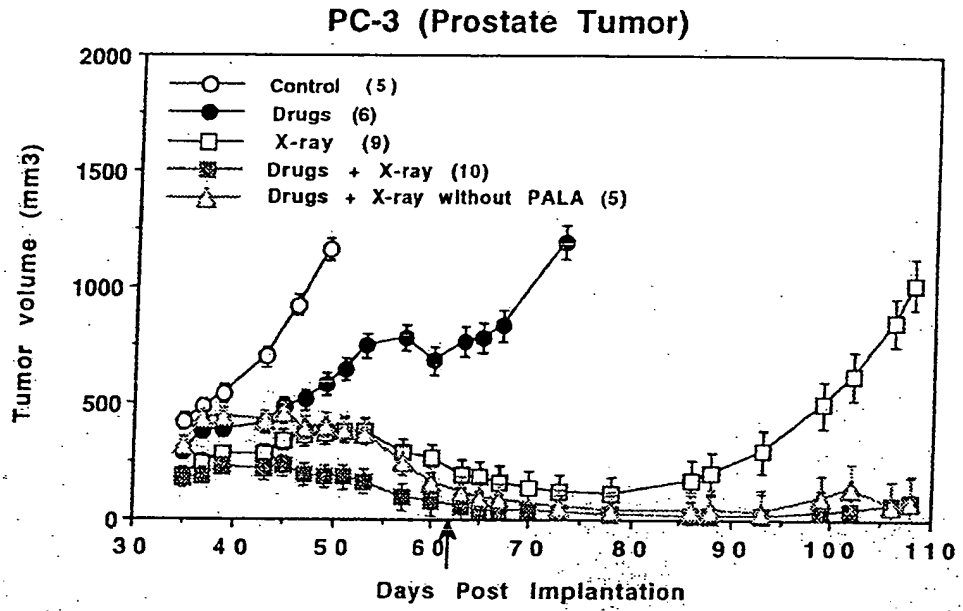


Fig. 2. The effect of CldC and its three biomodulators combined with radiation (the standard protocol [StP]) on the volumes of the prostate tumor PC-3. Treatment was for 4 weeks. The total dose of radiation was 47 Gy in 11 fractions. Also shown is the effect of omitting PALA. The final results of this experiment and all the experiments shown in Figs. 2–4 are summarized in Table 2. The numbers in parentheses indicate the number of tumors studied in each group. The average tumor volumes and the standard error are shown. In this figure and Fig. 3, the arrow indicates the last day of treatment postimplantation.

groups receiving X-ray alone and drugs plus X-ray, respectively.

Human glioblastoma SF-295

Only 3 weeks of treatment was used for the very rapidly growing human glioblastoma. The treatment was terminated, because it seemed that some of the tumors were responding to X-ray alone. This turned out to be a very transient response of 2/8 (25%) of the tumors. The total dose was 44 Gy in 8 fractions. The first day of treatment was only 13 days PI, and the last day was Day 31 PI. There was very little control by the drugs alone. Three of seven tumors treated with drugs plus X-ray were in tumor regrowth delay for more than 17 days, and one tumor in this group was cured, as indicated in Table 2. These responses seem remarkable in view of the rapid growth of this tumor.

Human breast tumor GI-101

This metastatic breast tumor was treated with a slightly protracted schedule of 3 weeks on, 1 week bye, and 2 weeks on. The total dose was 42 Gy in 14 fractions. The first day of treatment was 45 days PI, and the last day of treatment was 84 days PI.

As seen in Fig. 4, there is very little response to drug treatment alone, similar to results obtained with the glioblastoma described above. With full treatment, 7/11 (64%) tumors were fully controlled (<100 mm^3) for 30 days after cessation of treatment, whereas only 1/11 (9%) was controlled for that extended period in the group receiving X-ray alone.

The weight loss data are consistent with our finding with rodent tumors in immune-competent mice, that CldC coadministered with the biomodulators is not toxic at radiosensitizing doses. Similarly, in the immune-compromised mice of the present study, weight loss is no greater than that which occurred with radiation alone (Fig. 4b).

Human squamous cell carcinoma of the lung H-165

H165 tumors were treated for 4 weeks with 47 Gy in 12 fractions. The first day of treatment was Day 40 PI. Although the treatment ceased on Day 65 PI in mice treated with drugs plus X-ray, tumor control extended for 100 additional days in 3/5 (60%) mice, whereas with X-ray alone, only 1/5 tumors displayed an extended regrowth delay. As indicated in Table 2, 2/5 (40%) of the mice were cured with drugs plus X-ray, and 0/5 cures occurred with X-ray alone. There was no discernible effect on tumor growth by drugs alone, which abrogates any consideration of an additive effect of drugs and radiation.

Simplification of the protocol

The results of one of three experiments with PC-3 are shown in Fig. 5a–c. Whereas one group of mice was given escalating doses of CldC: 1035, 1160, 1245, and 1350 mg/kg in Weeks 1–4 (Condition D), another group of mice was given a constant loading dose totaling 1500 mg/kg each week via i.p. injections on Monday to Thursday for 4 weeks (Condition C). The total radiation dose for the 4-week period was 35 Gy. Those mice that were designated as cured in Week 7 received no further treatment. Mice that had

Table 2. Final results of the experiments shown in Figs. 2–4 and the experiment with the human glioblastoma SF 295 and the human lung tumor H-165 described in the text

Tumor, condition of irradiation	Condition	No. of tumors	Days to reach 4 × initial volume	Days in tumor regrowth delay	Fraction of cures
No. (Gy, Fractions, Weeks)	Control	5	14	0	0
Prostate, PC-3	StP*	6	28	0	0
(47, 11, 4)	X-ray	9	72	42	0
	StP + X-ray	10	116‡/124§	85‡/>97§	1/10
	Full,† No PALA	5	104/>142	56/>113	2/5
Prostate, H-1579	Control	5	13	0	0
(43, 11, 4)	StP	4	45	2	0
Pooled data	X-ray	8	24/>48	5/>29	1/8
	StP + X-ray	7	108/>134	81/>115	2/7
Glioblastoma SF-295	Control	5	3‖	0	0
(44, 8, 3)	StP	5	3‖	0	0
	X-ray	8	17	5	0
	StP + X-ray	7	31/>56	21/>51	1/7
Breast, GI-101	Control	7	26	0	0
(42, 14, 5¶)	StP	8	31	0	0
	X-ray	11	83	50	0
	StP + X-ray	11	100/>106	70/>80	1/11
Lung, H-165	Control	4	12	0	0
(47, 12, 4)	StP	3	20	0	0
	X-ray	5	58	32	0
	StP + X-ray	5	89/>133	72/>123	2/5

* StP, standard protocol (CldC, modulators, and schedule as shown in Table 1).
† The StP with PALA omitted.
‡/§ Calculations excluding cures/calculations designating cures as >200 days.
‖ For glioblastoma control and StP only; days to reach 2 × initial volume is shown.
¶ 3 weeks; 1 bye; 2 weeks.

visible tumors were treated in Week 8 and 10, as described in the legend of Fig. 5. The final frequency of cures was: AX) 0/10, BX) 3/9, CX) 4/10, and DX) 5/9. There were no additional cures as a result of the added treatment in Weeks 8 and 10 beyond those observed in Week 7; furthermore, two tumors in both Groups C and D, considered cured at the end of Week 7, grew after an extensive period of tumor regrowth delay. It is likely that greater tumor control could

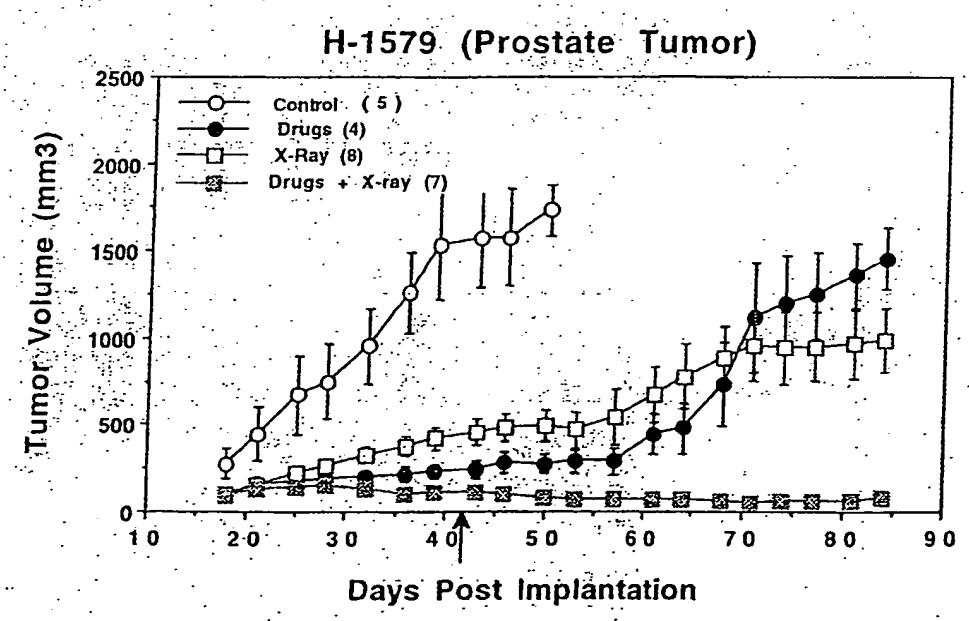
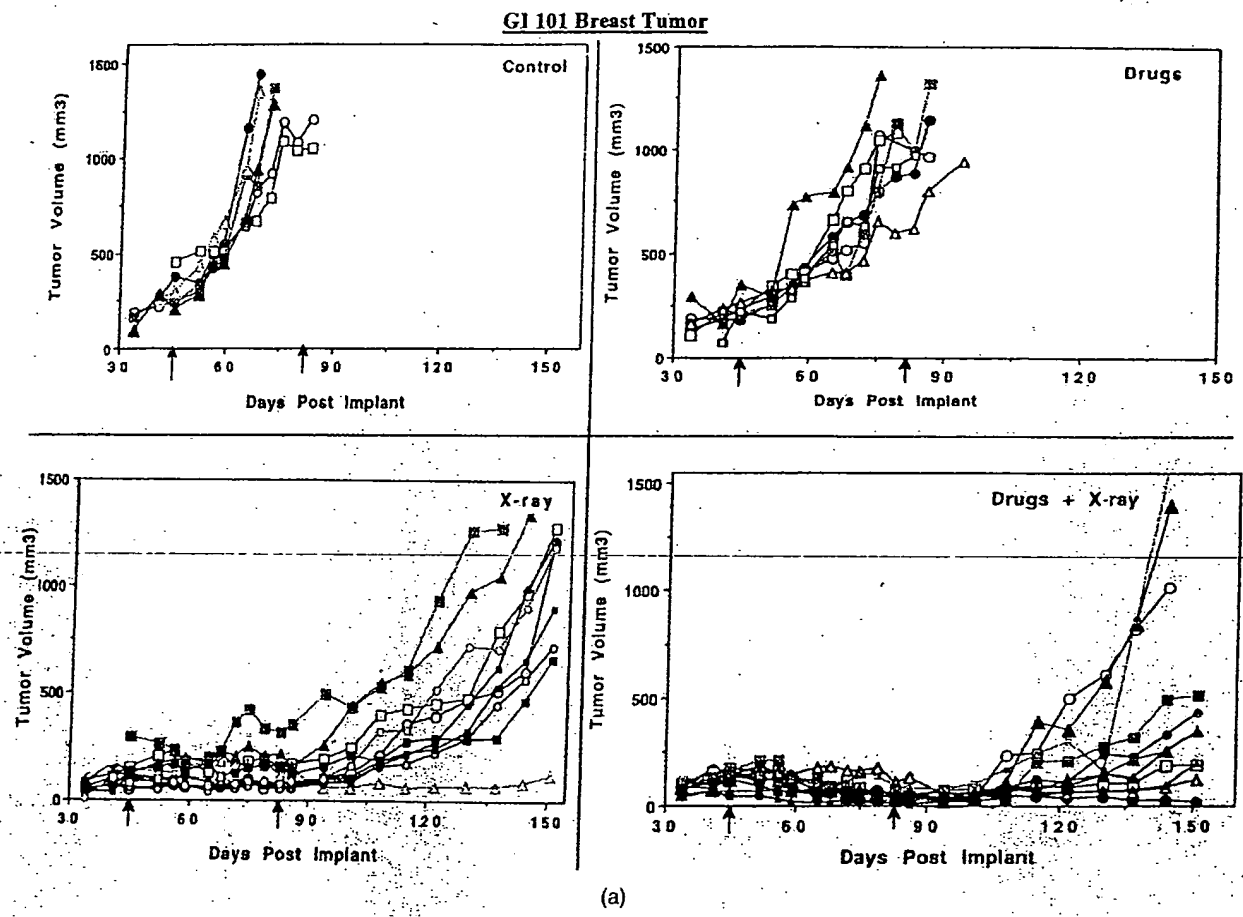
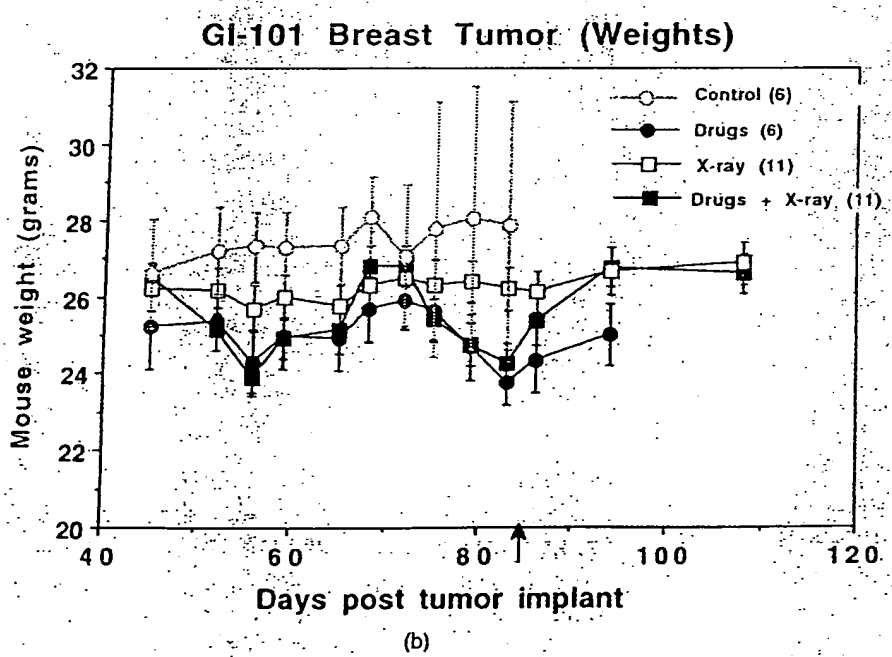


Fig. 3. The effect of CldC and its three biomodulators combined with radiation on the average volumes of the prostate tumor H-1579. Treatment was for 4 weeks. The total dose of radiation was 43 Gy in 11 fractions. See Fig. 2 for further explanations.

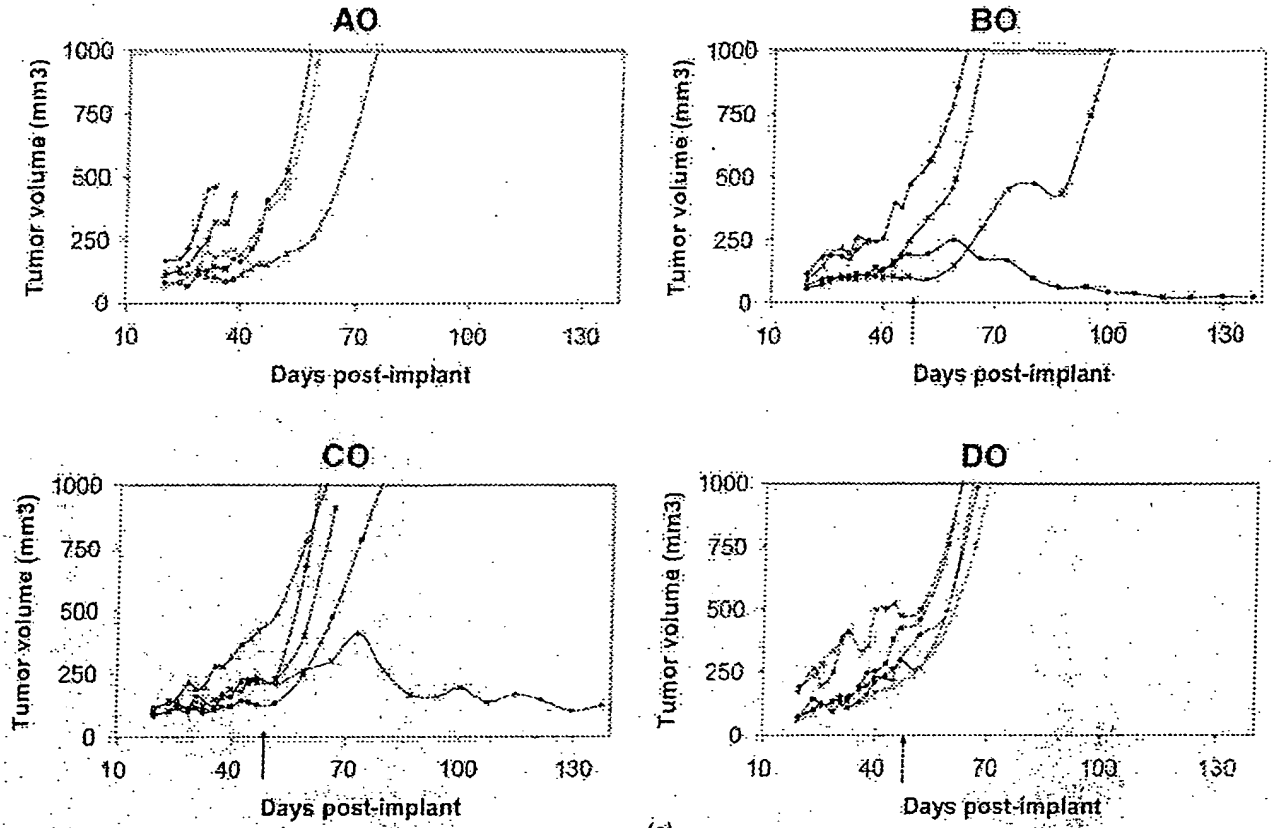


(a)

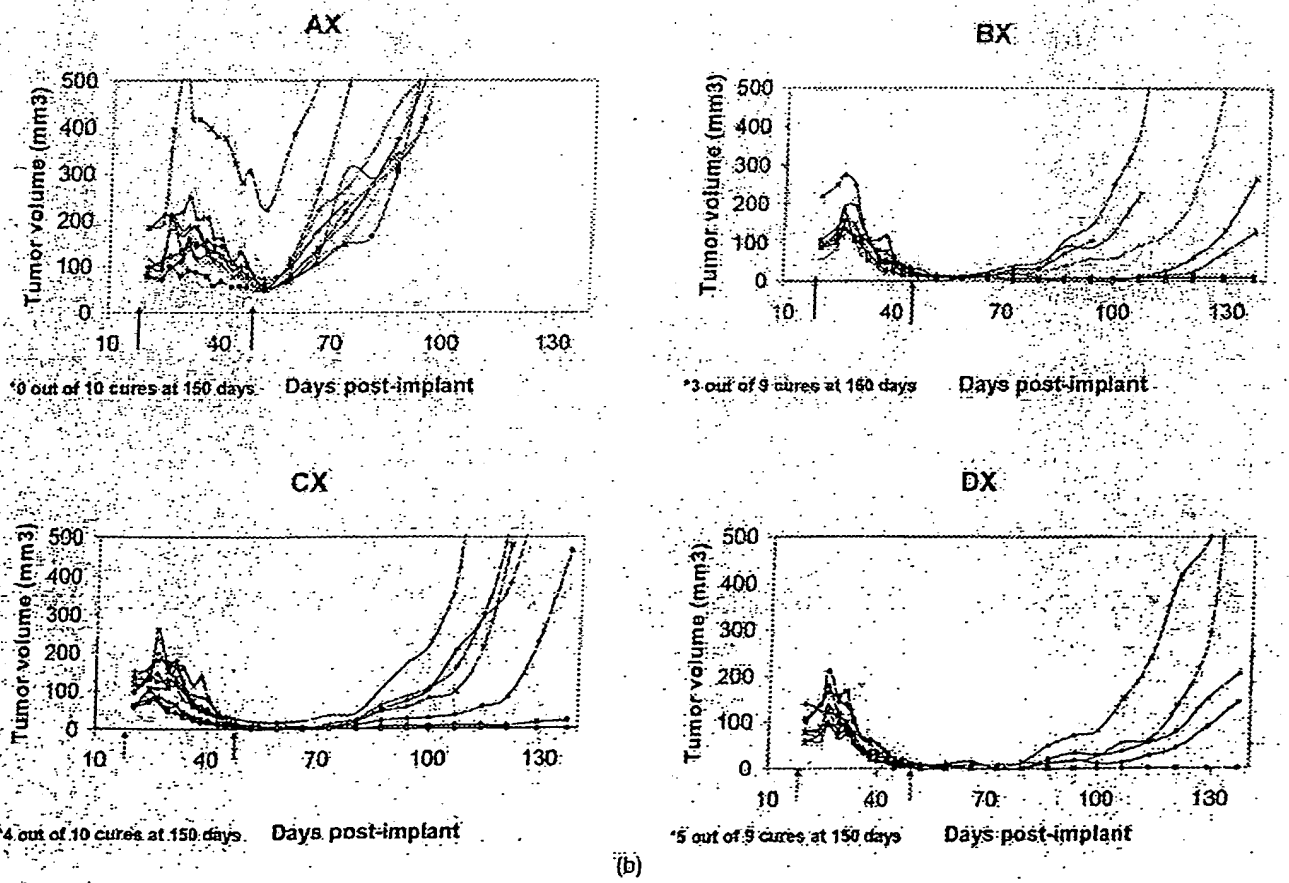


(b)

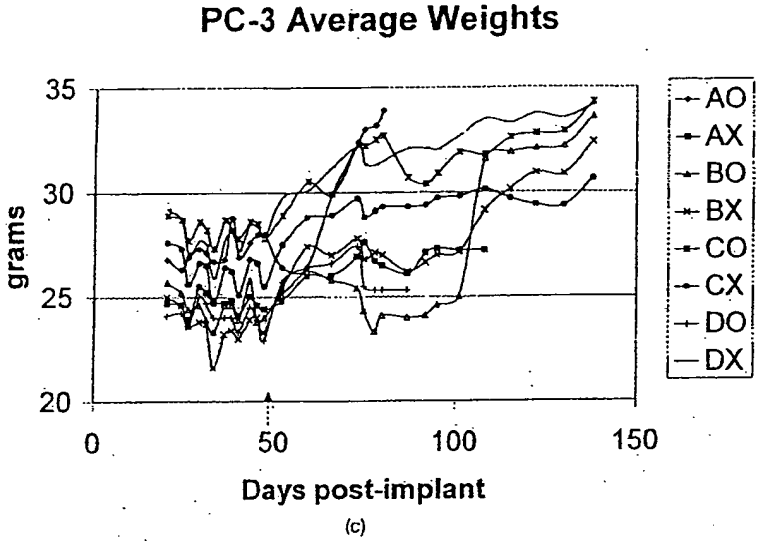
Fig. 4. (a) The effect of ClidC and its biomodulators combined with radiation (the StP) on the volumes of individual tumors of GI-101, a metastatic breast tumor. The treatment was for 3 weeks, followed by a "bye" of 1 week, followed by 2 weeks of treatment. The total dose was 42 Gy in 14 fractions. See Table 2 for the final results of this experiment and for the number of tumors in each group. The arrows indicate the first and last days of treatment. (b) Weight loss data: The average weights of each group are plotted, indicating the standard error. The arrow indicates the last day of treatment. The mice treated with drugs alone or with X-ray alone were sacrificed when their tumors approached ca. 1200 mm^3, hence the truncation of the lines representing these conditions.


AO
BO
CO
DO
Tumor volume (mm3)
Days post-implant
1000
750
500
250
0
10
40
70
100
130

(a)


AX
BX
CX
DX
Tumor volume (mm3)
Days post-implant
500
400
300
200
100
0
10
40
70
100
130
*0 out of 10 cures at 150 days
*3 out of 9 cures at 160 days
*4 out of 10 cures at 150 days
*5 out of 9 cures at 150 days

(b)



(c)

Fig. 5. A comparison of two simplified protocols utilizing only CldC plus H_4U with the standard protocol, in which CldC plus H_4U and two additional biomodulators are used, when combined with radiation of the human prostate tumor, PC-3. The volumes of individual tumors are plotted vs. days post tumor implantation. Schedule A: no drugs. Schedule B: the standard protocol (CldC plus 3 drugs as shown in Table 1). Schedule C: a loading dose for 4 weeks (1,500 mg/kg/week). Schedule D: an escalating dose of CldC (increasing ca. 10% each week); 1,035, 1,160, 1,245, and 1,350 mg/kg in weeks 1–4, respectively. Those mice not considered cured received 1,300 and 1,245 mg/kg in both weeks 8 and 10 in groups C and D, respectively. Mice not considered cured in group B received CldC plus the 3 drugs, as shown in Table 1, in weeks 8 and 10. The dose of H_4U was kept constant (25 mg/kg) in all conditions. (a) O: no radiation; the lines that end abruptly reflect groups of mice receiving no radiation that had to be killed because of their tumor burden. (b) X-radiation as described in text (a total of 35 Gy in weeks 1–4). The single arrow in Figs. 5a and c indicates the last day of the 4-week treatment. Those mice not considered cured were given a dose of 2Gy on Wednesday, Thursday, and Friday of weeks 8 and 10 for a total final dose of 47 Gy. Drugs were given in the a.m. and p.m. on Monday and Tuesday of each week, and only in the a.m. on Wednesday and Thursday. The tumors were irradiated as shown in Table 1, i.e., on the afternoon of Wednesday and Thursday and on Friday morning. (c) weight loss data.

have been achieved with continuous, rather than interrupted, weeks of treatment. The weight loss data shown in Fig. 5c indicates that, despite receiving injections of anesthetic 3×/week for 4 weeks and six injections of drugs/week, the treated mice recovered their weight loss each weekend, and their weight loss was no greater than that encountered with radiation alone.

Figure 6 summarizes the experiment with squamous cell carcinoma of the lung H-165, which was conducted to confirm that the simplified protocol was as effective as the protocol using four drugs. Tumor volumes on the first day of treatment were greater than usual. Initial tumor volumes usually averaged 120 mm^3; as in the experiment with this tumor, summarized in Table 2. In this study, the initial tumor volumes were 156, 151, 148, and 156 mm^3 in Groups AX, BX, CX, and DX, respectively. The total dose for 4 weeks of treatment was 52.5 Gy. After a bye of 3 weeks, treatment resumed on Day 51 to Day 60 with two courses of drug and radiation. The additional dose of radiation in the 10-day period was 22.5 Gy, resulting in a total dose of 75 Gy. The large arrow indicates the end of the initial 4 weeks of treatment. The smaller arrow indicates the initiation of the 10-day period of treatment. Note that there is no discernible effect of drugs on the growth rate of the tumors. The tumors of animals receiving radiation only were in regrowth delay only during the weeks they received radiation. The end points of the time to reach initial tumor volume were 47, 86, 92, and 95 days for irradiated groups AX, BX, CX, and DX, respectively. The end points of the time to reach 2× the initial tumor volume were 78, 89, 112, and 110 days. Clearly, the simplified protocols (Schedules C and D) are equal, if not superior, to the schedule using four drugs (Schedule B), as demonstrated with the prostate tumor PC-3. Despite a more aggressive, continuous 10-day period of treatment with drugs and/or radiation after a bye of 3 weeks, there were no cures. Figure 6b shows that the intensive 10-day treatment resulted in 10% weight loss in mice treated with radiation and Schedule D, a loss that was rapidly recovered. Again, one can suggest that continuous, aggressive treatment of the kind given to patients with head-and-neck tumors is more desirable than an interrupted treatment schedule, especially in view of the lack of appreciable weight loss, as shown in Figs. 5c and 6b.

Dosimetry of drugs and radiation

In an experiment using the human prostate tumor PC-3, we began an approach to determine the minimal effective dose of CldC using Schedule D with the doses of CldC used previously, as indicated in the experiment shown in Fig. 5. In addition, one group received one-half the dose of CldC (D/2). The experiment ran for only 4 weeks with H_4U

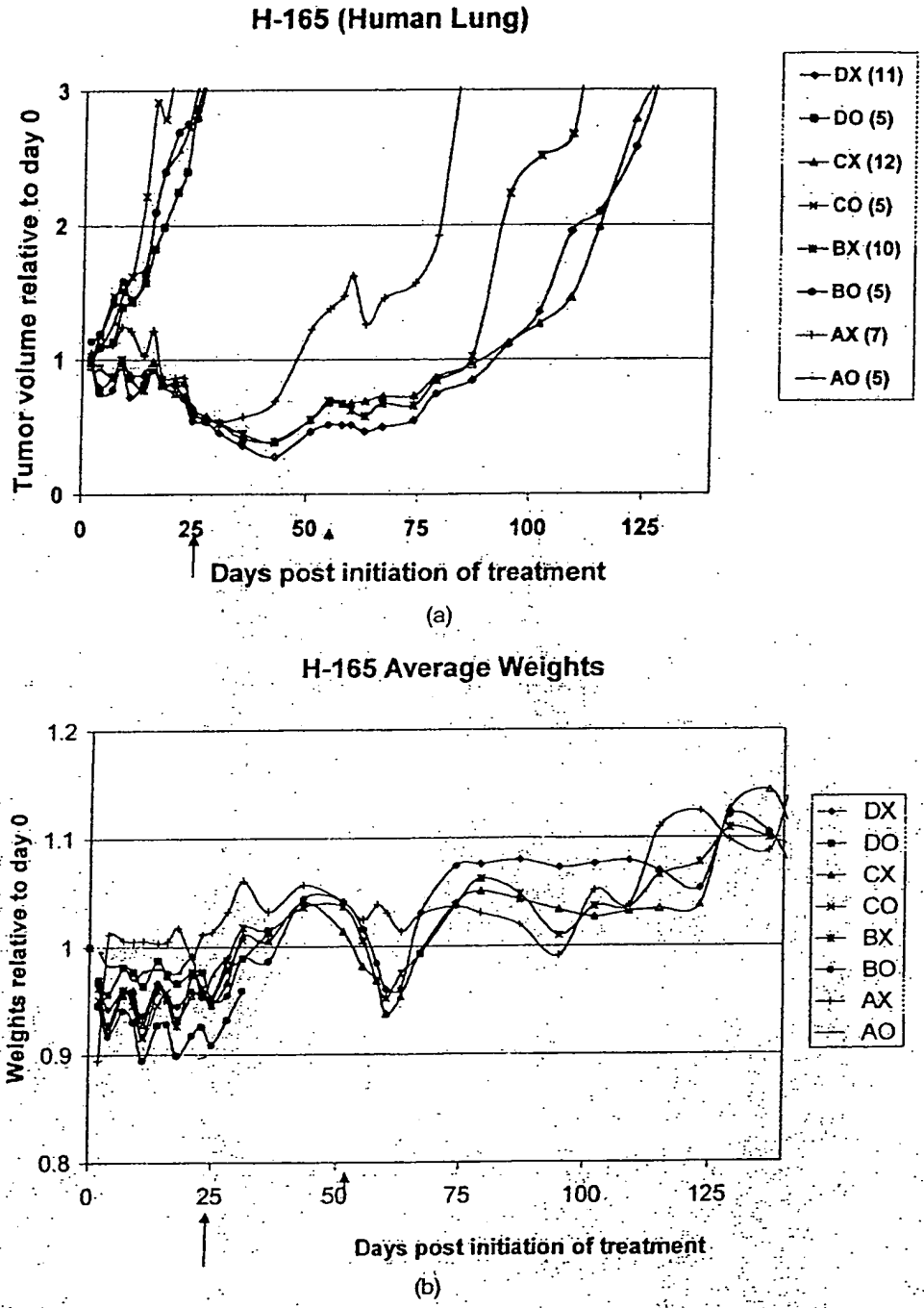
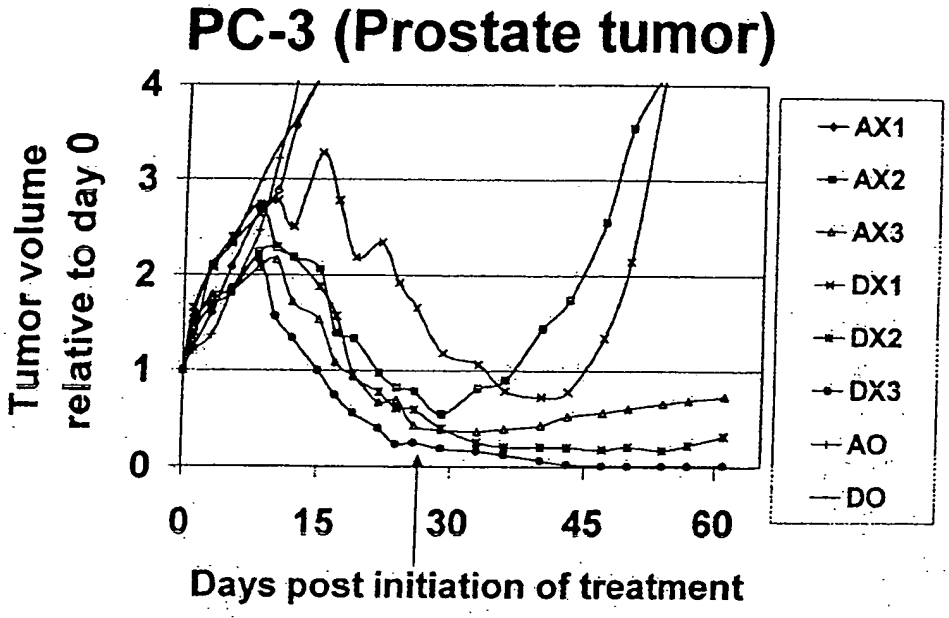


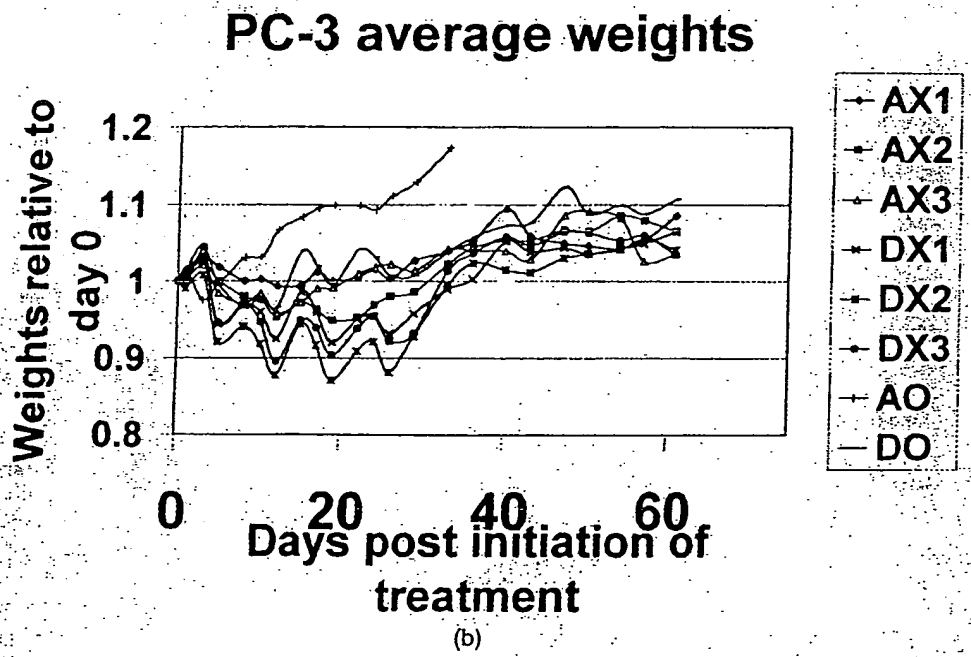
Fig. 6. (a) A comparison of Schedules C and D with Schedule B (as described in Fig. 5) using squamous cell carcinoma of the lung H-165. Because no cures were obtained, the data are best represented as average tumor volume relative to Day 0. The total X-ray dose for the 4-week period was 53 Gy. After a bye of 3 weeks, treatment resumed on Days 51–60 after the initiation of treatment. Two courses of treatment concentrated in a 10-day period gave an additional radiation dose of 22 Gy, resulting in a total dose of 75 Gy. Groups AO, BO, CO, and DO each had 5 mice per group. Groups AX, BX, CX, and DX had 7, 10, 12, and 11 mice per group, respectively, as indicated in the parentheses. The standard error of the mean was less than 10%. (b) Weight loss data.

always coadministered with CldC at a constant dose of 25 mg/kg. The total dose of radiation was 28.5 Gy.

The drugs without radiation had no effect on the growth rate of the tumors. The frequency of cures was 0/7, 2/9, and 6/10 for AX, D/2X, and DX, respectively. Tumor regrowth delay, not including cures, was 29, 47, and 65 days for AX, D/2X, and DX, respectively. Assigning a value of 200 days for cured mice, the data, including cures, was 29, 85, and 146 days for AX, D/2X, and DX, respectively. Thus the doses we are using in Schedule D apparently are close to the linear range with respect to tumor control. The dosimetric response we have obtained with the



(a)



(b)

Fig. 7. (a) A demonstration of the radiation dose increase effect of CldC plus H_4U with Schedule D, using the human PC-3 tumor. A = No drugs; D = CldC + H_4U as described for Schedule D in Fig. 5; O = no radiation; X1 = 13 Gy; X2 = 26 Gy; X3 = 39 Gy. Treatment was for only 4 weeks. The average tumor volume relative to its volume on the initial day of treatment is plotted against days post initiation of treatment. The arrow indicates the last day of treatment. (b) Weight loss data.

mouse model will help guide the design of studies that bring the findings to clinical practice.

Figure 7 summarizes an experiment in which we sought to determine the radiation dose increase effect of CldC + H_4U for groups with the PC-3 tumor and Schedule D after treatment for only 4 weeks. The total radiation dose for O, X1, X2, and X3 was 0, 13, 26, and 39 Gy, respectively.

The days in tumor regrowth delay for AX1, AX2, and AX3 are 9, 40, and 51; the days in tumor regrowth delay for DX1, DX2, and DX3 are 47, 59, and 60, respectively. These groups had seven tumor-bearing mice per group; groups AO and DO had four tumor-bearing mice per group. The standard error was generally less than 20% of the mean.

It appears that CldC + H_4U provides more than a twofold dose increase effect in that DX1 end points surpass those of AX2. In view of the results indicating that the tumors

receiving drugs and only 13 Gy were in tumor growth decline and regrowth delay longer than tumors receiving 2× the dose of radiation without drugs (Compare DX1 with AX2), it is striking that a total dose of only 13 Gy resulted in a dramatic reduction in tumor volume in 5/7 tumors and a potential cure! In contrast, the tumors treated without drugs and with 13 Gy grew at the same rate as untreated tumors (Compare AX1 with DX1). The weight loss data indicate that the moderate weight loss in drug-treated groups receiving radiation was substantially recovered each weekend and fully recovered once treatment stopped. After receiving essentially 1 Gy/fraction, all tumors in the DX1 group were less than their initial volume 18 days after the cessation of treatment; therefore, it is likely that substantial additional tumor control would have occurred if the treatment period had been extended for 3 more weeks without interruption, with a total dose of only 22 Gy. This suggestion has important clinical implications for the treatment of head-and-neck tumors which are usually treated 5 days/week for 6 or 7 continuous weeks with a total dose of 70 Gy.

Percent of PC-3 tumor cells incorporating 5-chlorouracil derived from CldC

In a collaborative study with Drs. Alan Pollack and Nicholas Terry of the MD Anderson Cancer Center, unirradiated mice bearing human PC-3 prostate tumors were treated with CldC + H_4U for 2 weeks as described for Schedule D. Using antibody vs. DNA containing ClUra and FACS analysis as described in publications from Dr. Pollack's laboratory (34), it was shown that the percent of cells of the tumor containing ClUra in their DNA was 51%, 78%, 92%, and 92% on Days 3 and 5 of Week 1 and Days 3 and 5 of Week 2, respectively.

Enzymatic studies

Table 3 summarizes the results of enzymatic studies with the normal and tumor tissue of 24 patients with head-and-neck tumors. The majority of the patients were previously treated with radiation. Both enzymatic assays could be conducted with as little as 40 mg of tissue. In three cases, equivalent tissue was not available. In 15/24 tumors, dCK is elevated greater than threefold (3–17×); in 12/24 tumors, dCMPD is elevated greater than sixfold (6 to 140×); and in 18/24 patients, dCMPD is elevated threefold or greater. In 11/24 tumors, both enzymes are elevated greater than threefold. The elevation of dCMPD is most critical for the strategy. Starting with Tumor 14, the study was conducted as a blind study because of the remarkable nature of the results. In the paper by Guisti *et al.* (28), on which this strategy is based, two malignant head-and-neck tumors were also found to have high levels of dCMP deaminase: carcinoma of the buccal mucosa and adenocarcinoma of the floor of the mouth. These levels were approximately 7× higher than the levels in the following benign tumors: fibroma of the gum and lips, papilloma of the palatine mucosa, and chrondroma of the jaw.

It should be noted that in one patient, with respect to dCK, and in two patients, with respect to dCMP deaminase, the large factor of increase is due to a very low level of enzyme activity in adjacent normal tissue rather than to an unusually high level of activity in the tumors.

Cell cultures of two human squamous cell carcinomas of the head and neck were examined enzymatically before future radiation studies in the nude mice model: SCC-1 and SCC-6, a tumor of floor of the mouth and of the tongue, respectively. The levels of dCK were 198 and 143 pmoles/min/mg protein, and the levels of dCMPD were 87.4 and 81.0 nmoles/min/mg protein for SCC-1 and SCC-6, respectively. The rates of phosphorylation of dC to dCMP and the rate of conversion of dCMP to dUMP were similar to the high rates obtained with the tumor of Patient 10 in Table 3, for example; however, the low level of protein in cell culture extracts compared to that of human tissue contributes to the extremely high apparent enzyme activity observed.

The levels of dCK in the PC-3 human prostate tumor and H-165, the human squamous cell carcinoma of the lung, are 16.2 and 35.5 pmoles/min/mg of protein, respectively. The levels of dCMP deaminase in the PC-3 and H-165 human tumors are 3.63 and 14.1 nmoles/min/mg of protein, respectively. With PC-3, the levels of both dCK and dCMPD are 3× higher than more than 1/3 of the normal tissues shown in Table 3. In the H-165 tumor, the levels of dCK and dCMPD are more than 3× higher than 1/2 and 3/4, respectively, of the normal tissues shown in Table 3.

Normal tissue damage

Nude mice underwent histologic examination of the cervical spinal cord at 10 weeks and at 5 months after cessation of irradiation limited to that area. One-half of the mice received CldC + H_4U for 4 weeks in accord with Schedule D. All mice were irradiated, with one-half receiving a total of 30 Gy, the other half receiving a total of 48 Gy. Neither paralysis nor treatment-related damage was observed. The histologic studies revealed 4/17 and 6/20 minor microglial nodules in the X-ray only and X-ray plus drug group, respectively. Dr. Carol Petito, the study pathologist, considers these minor aberrances to be unrelated to the treatment.

DISCUSSION

In our past studies with immunocompetent mice, CldC and three biomodulators were shown to be efficacious vs. 5 rodent tumors: RIF-1, a nonimmunogenic radiation-induced fibrosarcoma; Sarcoma-180; Lewis lung carcinoma; a DMBA-induced mammary adenocarcinoma (35, 36); and EMT-6 (18), another mammary adenocarcinoma for which 60–80% cures were obtained and a threefold dose increase effect was demonstrated with weight loss no greater than that obtained with radiation alone.

CldC is a prodrug that is modified by tumor physiology with the potential to become a powerful selective radiosensitizer of human malignant tumors. Although there are only two "minor" differences between CldC and 5-bromo- or iodo- deoxyuridine (the halogenated nucleoside analogs in

Table 3. A summary of the levels of dC kinase and dCMP deaminase in the head and neck tumors and associated normal regions of patients with tumors of different histological classification at several sites and various stages.

					Deoxycytidine Kinase			dCMP Deaminase		
Patient	Site	Stage	Histology	Tumor Normal	Mean	SD*	T/N	Mean	SD	T/N
1	Larynx	IVT4N0	SCCA	T	10.4	*0.56*	1.4	18.3	*3.0*	30
				N	7.45	*0.35*		0.619	*0.13*	
2		IIT2N0	SCCA	T	22.7	*0.34*		14.1	*4.6*	
				N						
3		IIIT3N0	SCCA	T	61.6	*0.35*	2.3	49.1	*6.9*	5.8
				N	26.9	*3.6*		8.53	*0.93*	
4		IVT4N2	SCCA	T	52.4	*12*	2.8	22.0	*1.7*	9.5
				N	18.9	*1.1*		2.31	*0.36*	
5		IT1N0	SCCA	T						
				N	3.60	*0.50*		4.90	*2.2*	
6		IIIT3N0	SCCA	T	39.5	*4.2*	1.2	18.9	*0.88*	2.9
				N	32.1	*10*		6.43	*0.94*	
7		II (rT2N0)	SCCA	T	16.0	*5.6*	0.71	19.3	*2.2*	4.8
				N	22.6	*6.9*		4.06	*1.1*	
8		III(T3N0)	SCCA	T	52.2	*5.6*	3.1	16.7	*3.1*	3.1
				N	17.1	*0.49*		5.43	*2.2*	
9	Oral Cavity	IVT4N0	SCCA	T	15.1	*4.2*	3.1	8.78	*1.2*	16
				N	4.90	*0.42*		0.537	*0.005*	
10		IVT1N2	SCCA	T	64.4	*6.2*	4.6	26.8	*2.9*	9.7
				N	14.0	*4.5*		2.77	*1.1*	
11		IIIT2N1	SCCA	T	26.1	*2.2*	17	17.5	*3.3*	20
				N	1.52	*1.1*		0.889	*0.52*	
12		(IIIT1N2b)	SCCA	T	40.4	*5.9*	2.4	12.8	*1.9*	1.9
				N	17.0	*7.4*		6.73	*1.4*	
13		IV(T2N2b)	SCCA	T	22.2	*49*	1.36	11.8	*0*	12
				N	16.3	*4*		0.984	*0.002*	
14		IV(T4N0)	SCCA	T	121	*38*	7.1	117	*4.2*	2.2
				N	17.1	*8.6*		53.6	*6.8*	
15		IVT1N2B	SCCA	T	20.0	*2.1*	2.6	11.5	*1.4*	2.5
				N	7.71	*2.2*		4.60	*0.53*	
16	Tongue	IIT2N0	SCCA	T	143	*0.92*	11	27.0	*4.5*	4.4
				N	13.0	*3.1*		6.08	*0.63*	
17		IVT2N2B	SCCA	T	29.1	*0.14*	5.1	10.4	*0.85*	6.1
				N	5.70	*0.30*		1.70	*0.39*	
18	Oropharynx	IIT2N0	SCCA	T	42.0	*2.3*	9.4	20.1	*2.5*	26
				N	4.45	*0.24*		0.788	*0.40*	
19	Maxilla	IVT4N0	SCCA	T	43.3	*10*	1.9	16.9	*0.42*	3.7
				N	23.3	*4.5*		4.50	*0.31*	
20	Parotid	IVT4N0	Basi-squamal carcinoma	T	27.3	*6.0*	16	6.63	*2.4*	8.4
				N	1.71	*0.50*		0.785	*0.42*	
21	Submandibular	NA	NA	T	45.7	*6.2*	9.5	37.1	*3.5*	20
				N	4.80	*0.78*		1.90	*0.71*	
22	Facial Skin	IIIT3N0	SCCA	T	31.6	*8.2*	4.1	21.8	*2.6*	2.2
				N	7.80	*1.1*		10.1	*0.78*	
23	Pharynx Tonsil	IIIT3N0	SCCA	T	23.8	*3.3*		17.6	*5.1*	
				N						
24	Hypopharnyx	IIT2N0	SCCA	T	26.2	*0.78*	5.5	10.2	*2.5*	2.7
				N	4.70	*2.3*		3.78	*1.8*	
25	Thyroid	IVT4N0	Anaplastic	T	6.40	*2.5*	1.1	7.49	*3.5*	2.4
				N	5.61	*0.20*		3.10	*1.4*	
26		IVT4N1B	Papillary	T	101	*7.1*	4.8	27.6	*2.0*	140
				N	21.0	*3.0*		0.198	*0.057*	
27		IIT2N0	Papillary	T	112	*6.0*	5.6	32.0	*13*	1.6
				N	20.0	*5.0*		19.7	*3.1*	

T: tumor, N: tissue from normal margins; dC kinase: pmoles/min/mg protein; dCMP deaminase: nmoles/min/mg protein; SD*: Standard Deviation of the mean. †The majority of these patients were previously treated with radiation. N (under stage): nodes; r recurrence.

recent use as radiosensitizers), these modifications seem to result in major increases in efficacy as determined in our past studies using IdU with EMT-6, a rodent mammary adenocarcinoma (18), and in a study using IdU tested at its maximum tolerated dose with PC-3 (data not shown). CldC, when anabolized to CldCMP by deoxycytidine kinase and converted by dCMP deaminase to CldUMP, is further anabolized and incorporated selectively into tumor DNA as CldU (35) (See Fig. 1). When tumor DNA containing CldU is irradiated with X-ray, the Cl atom is released, and a uracyl radical is formed that results in persistent single-strand breaks in tumor DNA. It is presumed that the repair capacity of the tumor cell is overrun, and the cell dies. The elevation of dC kinase in many human tumors is consistent with the observed predominance of salvage pathways in tumors. The elevation of dCMP deaminase has been shown to be the result of the loss of end-product inhibition by TTP in a tumor in a single study (31). As much as 40% thymidine in DNA is derived by the dCMP → dUMP (dCMP deaminase pathway) (37). dCMP deaminase also prevents the reutilization of CH_3dCMP, which is formed by DNA degradation via DNase in successful solid tumors that become necrotic. Salvage of CH_3dCMP would result in the silencing of genes, which would be detrimental to the tumor (38). dCMP deaminase converts this metabolite to TMP, important for tumor metabolism as described above. Thus, this technology exploits the elevation of these enzymes (which play an important role in successful tumor physiology) for a therapeutic advantage.

If the substitution at the 5 position is Br or I, the deoxycytidine analog is a poor substitute for dC kinase (400 and 1000 μM Kms compared to a Km of 56 μM for CldC, respectively) (39, 40). If a keto rather than an amino group is in position 4, as with BrdU or IdU, the enzyme-based selectivity is lost, and incorporation takes place in rapidly growing tissue such as bone marrow and intestine. H_4U, which is effective at 1/400 its toxic dose (41), prevents premature systemic deamination. In view of our finding that H_4U inhibits the cytidine deaminase in tumors to a far lesser extent than in normal cells (42), a secondary distinct pathway of metabolism (via thymidine kinase) is available for the formation of CldUMP in tumor cells (See Fig. 1). This dual pathway activation of CldC (and FdC) makes it unlikely that mutation to resistance to X-ray will occur.

In contrast to IdU and BrdU, dC and its analogs are not catabolized by uridine and thymidine phosphorylases (43), and the nucleotide derived from it, CldUMP, is not dehalogenated by thymidylate synthetase (44). Systemic catabolism by uridine and thymidine phosphorylases greatly limits the effectiveness of the halogenated analogs of deoxyuridine (43). These three enzymes are elevated in tumor cells (45–47). The pathway of conversion toward tumor radiosensitization is favored because the Kms of dCMP and dCDP kinases are very high (48, 49). The conversion of CldCMP to CldUTP likely results in the activation of dC kinase (based on studies with TTP), whereas IdUTP and BrdUTP inhibit their key initial activating enzyme, thymidine kinase. Therefore, CldC may enhance its own incorporation into DNA. In addition, CldUTP formed from CldC likely inhibits nucleoside diphosphate reductase, as inferred from studies with BrdUTP (50). This also results in CldC autoenhancing its own incorporation into DNA by lowering the competing pools of TTP. The nucleotide pool imbalances caused by inhibition of the reductase has been shown to result in tumor-directed apoptosis and single-strand breaks equivalent to that which would be obtained with 20 Gy (51).

The results with CldC + H_4U (Schedules C and D) in experiments with PC-3 and H-165 shown in Figs. 5–7 are encouraging, because the prevailing view of this technology has been that, despite its success vs. rodent tumors, the use of four drugs presents problems in its moving rapidly to clinical application. It seems that the addition of two inhibitors of the formation of TTP at *both* an early step (aspartyltranscarbamoylase) *and* a late step (thymidylate synthetase) may be necessary only for the rapidly growing rodent tumors. The high percent (92) of the cells of the PC-3 tumor incorporating CldU derived from CldC (+H_4U) after only 1½ weeks of treatment may explain the marked radiosensitization we have observed, especially if there is a reasonable extent of replacement of thymine by 5-chlorouracil in these cells (studies in progress).

Because of the intermediary Van der Waal radius of the Cl atom and its strong electronegativity, CldC may play multiple roles in the strategy of radiosensitization. This duality of CldC may explain its similarity to IdU and BrdU as a radiosensitizer and as a candidate inhibitor of nucleoside diphosphate reductase in one regard; its similarity to FdC may contribute indirectly to its effectiveness as a radiosensitizer. High levels of CldUTP formed from CldC may not only overrun the competition of TTP, but CldUMP may effectively inhibit thymidylate synthetase. The fact that FdUMP and CldUMP are not dehalogenated by thymidylate synthetase, whereas BrdUMP and IdUMP are dehalogenated (44), may be consistent with this suggestion. Indeed, studies with the thymidylate synthetase of *Lactobacillus casei* in the laboratory of Wataya and Santi (52) have shown that the Ki of CldUMP was far greater than FdUMP but much less than that of BrdUMP and IdUMP (Ki FdUMP = 0.015, CldUMP = 0.19, BrdUMP = 1.4, IdUMP = 1.6).

Brief chemical studies with the thiol bisulfite dispel the prevailing view that CldC is just another predictable 5-halogenated pyrimidine analog. Whereas 5-iododeoxyuridine and 5-iododeoxycytidine are dehalogenated by bisulfite, CldU is not modified; furthermore, bisulfite, which is known to deaminate deoxycytidine without deaminating 5-methyl deoxycytidine, does not dehalogenate or deaminate the unique pyrimidine analog CldC (A. Mian, Ph.D. and S. Greer, unpublished data, July 2000).

Other areas of study need to be pursued that may point to CldC enhancing its own radiosensitization and complementing radiation-induced tumor cure by mechanisms that are attributes of FdC and its metabolites. Is CldU in DNA a

substrate for uracil-N-glycolyase? This could invite additional DNA breaks by the apurinic/apyrimidinic endonuclease that could follow the glycolyase, thereby increasing the burden of repair. Is CldC in DNA an inhibitor of DNA 5-cytosine methyltransferase, thus unsilencing tumor suppressor genes or invasion suppressor genes (e.g., Cadherin), resulting in return of tumor cells that survive radiation to a normal state or one incapable of migrating to distant sites or one with restored DNA repair capacity, initially silenced by hypermethylation? Can CldC, via hypomethylation, remove hotspots of mutation, resulting in a return of a genetically unstable tumor cell that survived radiation to a genetically stable cell that is less likely to be more aggressive, metastatic, or refractory to further treatment? Can hypomethylation by CldC result in the expression of silenced tumor surface antigens in cells surviving radiation death so that the patient's immune system will be recruited to play a role in efficacy? Although we have found very little CldC as such in tumor DNA (35), because of the elevated Kms of the higher nucleotide kinases (48, 49), coupled with the action of elevated dCMP deaminase in tumors (28), it would nonetheless take very little (nonstoichiometric) incorporation of CldC into DNA to inhibit the processive enzyme 5-cytosine DNA methyltransferase. The answers to these questions will impact on the conduct and outcome of clinical studies with CldC as a radiosensitizer of human tumors.

The first trial will examine this strategy vs. tumors of the head and neck, which usually are treated with 7 uninterrupted weeks (once/day, 5 days/week) of radiation therapy. When biopsy material is readily available, as is the case with head-and-neck tumors, an enzyme profile can easily be determined to predict the potential responsiveness of a tumor to radiation with CldC. Adding to the potential of CldC as a radiosensitizer of human tumors is the finding (53) that radiation increases the levels of pyrimidine salvage enzymes such as dCK. Tumors other than those of the head and neck that have elevated levels of dCMP deaminase, such as glioblastoma, which has a 125-fold higher level of the enzyme than surrounding brain cortex (28), will be compelling targets for further clinical trials, especially in view of the ability of deoxycytidine and its analogs to cross the blood–brain barrier (54).

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